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Investigating the proteomic profiles of cattle hide resulting in loose and tight leather throughout early processing stages

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1. Introduction

Leather is a durable and flexible material that is made by tanning degradable animal skins or hides, to produce a material that is stable, and no longer subject to bacterial degradation [1]. Looseness is a defect found in cattle hide that causes a wrinkly appearance in the finished leather when subjected to certain forces and subsequently results in reduced leather quality [2-4]. Previous studies have investigated looseness in cattle hide using a combination of microscopy techniques [2, 4], small angle X-ray scattering [2], ultrasonic imaging [3] and Raman spectroscopy [5] using wet blue or finished leather samples. Studies by Wood and Wells *et al.* [2, 6] showed a larger separation between the fibre bundles in loose leather. Wells *et al.* and Liu *et al.* [2-4] reported that loose leathers have a gap between the grain and corium layers that is absent in tight leathers and Mehta *et al.* [5] detected differences in protein and lipid Raman fingerprints in loose and tight wet blue samples. Defects in the hide may be a result of its environment, e.g. scarring and insect infestation; the preparation of hides for tanning e.g. flay-cuts and gouges, putrefaction and heat damage; or poor tanning practices during tanning processing such as over liming or too much proteolytic enzyme [7, 8]. It is also possible that defects are due to a change in the molecular components of hide caused by poor nutrition, stress, disease or genetic factors [9].

Hides consist of many different macromolecules with the most abundant being proteins. These proteins aid in the multitude of functions that hide performs, including scaffolding, elasticity and strength [10-12]. During the early stages of leather processing many of the non-collagenous proteins are removed, these processes which include removing the hair, removal of non-collagenous material and opening up of the collagen fibres influence the final properties of the leather including its quality [1, 13]. Collagen, elastin, proteoglycans and glycoproteins are the predominant proteins that are discussed in relation to leather, due to the fact that they are the

most prevalent and/or have significant and known impact on the quality of the leather [1, 7]. However, a wide range of other proteins are present in hide that have come to light in recent years with the growing capabilities of proteomic techniques. These techniques have been utilised to show that a wide range of proteins are affected by factors including skin diseases, ageing and stress [14-17].

This study investigated the proteomic profiles of cattle hide throughout the early stages of leather processing – raw, lime, delime & bate and pickle. Gel liquid chromatography with tandem mass spectrometry (gel LC-MS/MS) was used to investigate the proteomic profile of loose and tight cattle hide. Although there are many different methods available to study proteins in hide including western blotting, 2D gel electrophoresis and MS this study used LC-MS/MS because it is highly sensitive and specific, and enables large-scale analyses of biological systems [18, 19]. This study will help to discover the correlation between the proteomic profile of cattle hide and the leather defect looseness.

2. Experimental

2.1 Chemicals

All chemicals used for trypsin digestion and analysis were mass spectrometry grade (Optima® LC/MS) chemicals purchased from Fisher Scientific. Exceptions to this include; MS grade Trypsin Gold purchased from Promega; Wisconsin, USA, cOmplete® protease inhibitor tablets from Roche Diagnostics; Mannheim, Germany. DL-Dithiothreitol (DTT) from Gold Biotechnology; USA and iodoacetamide, urea and thiourea from GE Healthcare; Buckinghamshire, UK. Coomassie blue G-250 and 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate (CHAPS) from Biorad; California USA. Glass beads (acid washed) were purchased from Sigma Aldrich; St. Louis, USA. All other chemicals were analytical grade.

2.2 Sample Preparation

Four random hides that had already been removed from the carcass were obtained from Tasman Tannery, Whanganui, NZ. The hides were labelled and cut in half, with one half being processed to finished leather using a standard processing procedure at New Zealand Leather and Shoe Research Association (LASRA) to assess looseness [2] whilst the other half was sampled throughout the early processing stages including raw hide, liming stage, delime and

bating stage and pickled stage in both the OSP (tight) and distal axilla (DA) (loose) regions of hide as seen in Figure 1. These samples were stored at -20 °C for later analysis.

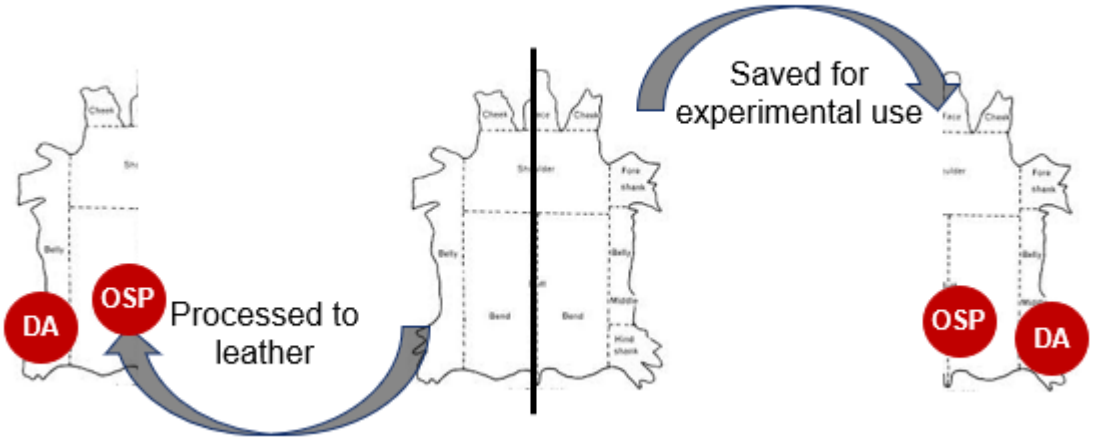

Figure 1. Sample preparation of cattle hide showing loose (DA) and tight (OSP) regions.

Looseness was measured using the SATRA STD 174 break/pipiness scale (SATRA Technology; Northampshire, UK) which consists of a graded selection of leather replicas numbered one to eight with one having the least severe wrinkles and eight having the most severe as seen in Figure 2.

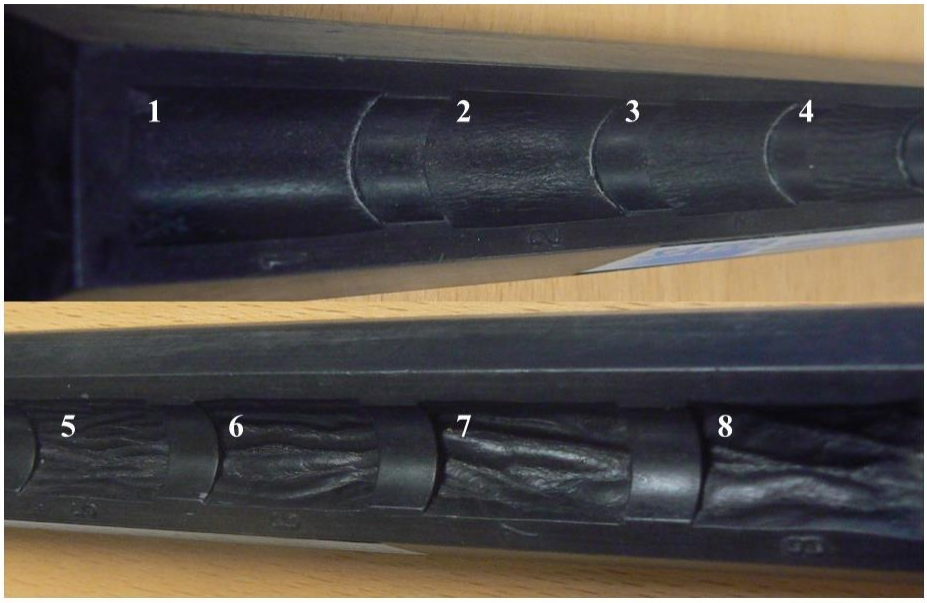

Figure 2. SATRA STD 174 break/pipiness scale.

2.3 Protein extraction

Samples were cut using the Leica CM 1850 UV cryostat (Leica Biosystems; Wetzlar, Germany) to 10 µm thick sections and approximately 50mg of hide were separated into grain, middle and corium sections as seen in Figure 3. The samples were placed in 1.5 mL Eppendorf tubes then immersed in lysis extraction buffer (7M Urea, 2M thiourea, 40mM DTT, 4% CHAPS, 30mM tris and 1x cOmplete® protease inhibitor tablet used according to the manufacturer's instructions, pH 7-9) for 24 hours at 4°C. The extraction was aided by mechanical action provided through adding glass beads to each tube which was then placed on a rotating wheel (LABNET, USA) overnight. After this time, residual hide was removed from the protein solution by centrifugation at 16,500 x g for 30 minutes. The pellet was then treated with a second lot of extraction buffer as shown in Figure 4. The supernatants from both lysis buffer extractions were pooled as were the supernatants from the sequential extraction and the proteins precipitated by the addition of 25 % TCA in acetone in a (v/v) ratio of 1:9. After incubation at -20°C for at least 2 hours precipitated proteins were pelleted by centrifugation at 5,000 x g for 20 minutes, and the resulting pellets washed 3 times in cold acetone before being resuspended in the minimum volume of sample solution (7M Urea, 2M thiourea, 40mM DTT, 4% CHAPS and 1x cOmplete® protease inhibitor tablet, pH 7-9).

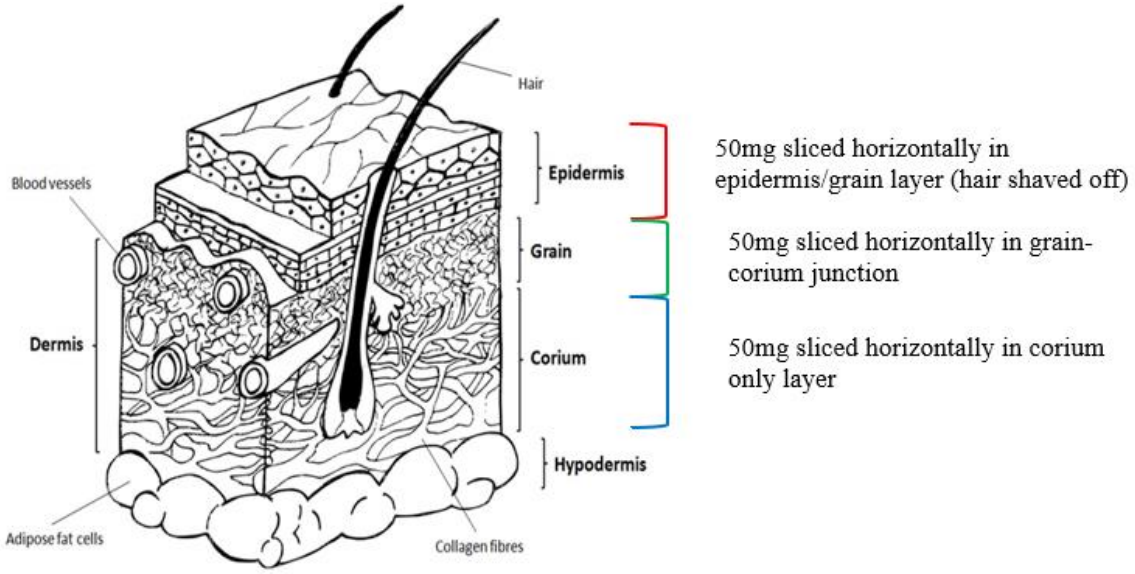

Figure 3. Diagram illustrating how the samples were cut prior to protein extraction.

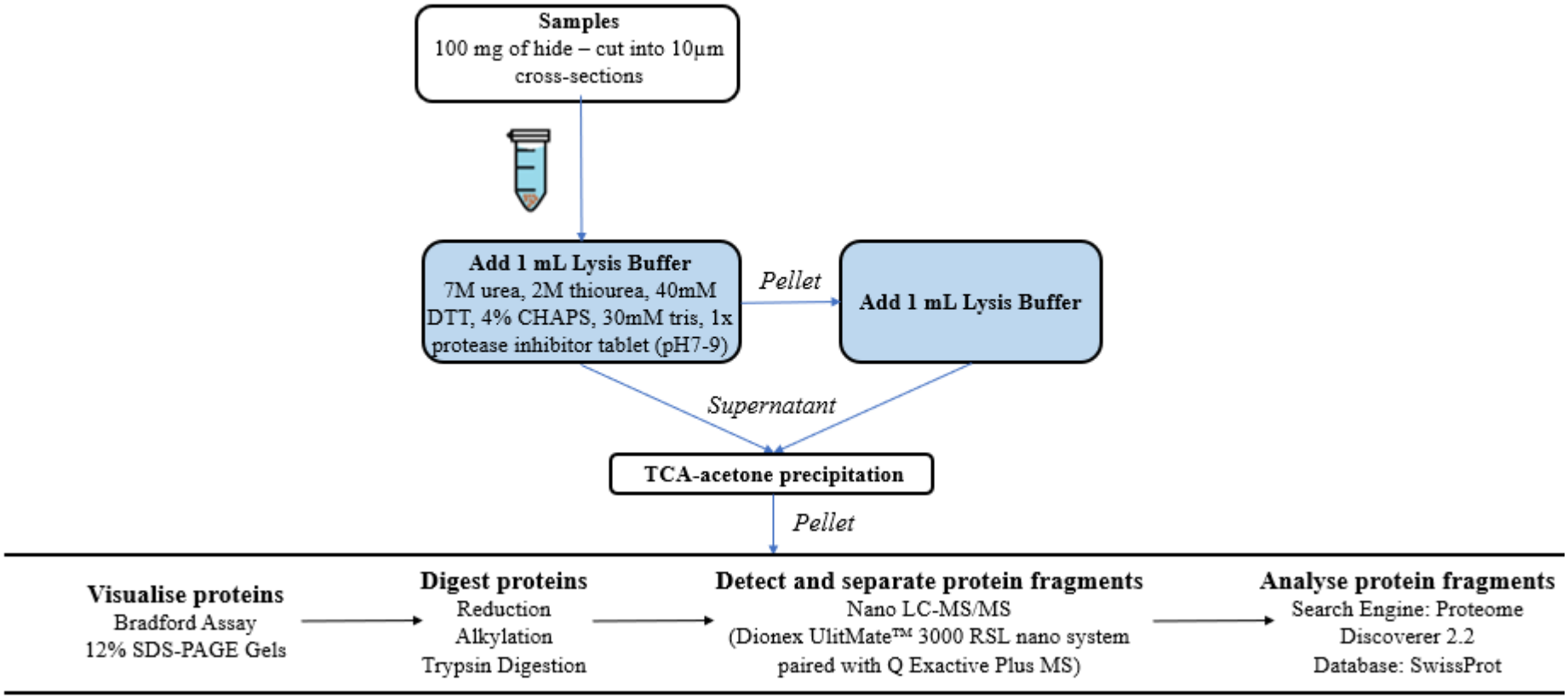

Figure 4. Flow diagram of protein extraction from cattle hide samples followed by in-gel tryptic digestion and LC-MS/MS analysis.

2.4 Protein digestion

The concentrations of the samples were measured using the standard Bradford assay protocol [20]. An equal volume of sample was mixed with the same volume of sample loading buffer (10% (v/v) SDS, 50% (v/v) glycerol, 100mM DTT, 0.25M Tris-HCL, 0.05% (w/v) bromophenol blue) and run on 12% Tris-glycine SDS-PAGE gels at 150V for approximately 90 minutes alongside precision plus protein^{TM} dual xtra standards ranging in molecular weight from 250kDa to 20kDa from BioRad. Following electrophoresis, the gels were fixed in ethanol: acetic acid (40:10 (v/v)) for 15 minutes before being stained overnight with Colloidal Coomassie brilliant blue G250 [21].

Each lane was manually cut out of the gel using a sterilised scalpel blade then sliced into 6 even pieces. After cutting each band into small pieces, they were destained using 50% methanol, 5% acetic acid and dehydrated in 200µL acetonitrile. The gel pieces were air dried before being reduced by the addition of 50µL of 10mM DTT in 100mM ammonium bicarbonate. After 1 hour at room temperature, the solution was removed and replaced with 50µL of 200mM iodoacetamide in 100mM ammonium bicarbonate and the tubes incubated for 1 hour at room temperature in the dark. After this time the alkylating solution was removed, and the gel pieces washed in acetonitrile and dehydrated as before. They were then rehydrated and subjected to in-gel digestion with 6 µL 100µg/mL MS grade Trypsin Gold in 50 µL ammonium bicarbonate, 1mM $CaCl_2$, 10% (v/v) acetonitrile at 37 °C overnight. The supernatant was carefully removed from the gel pieces and placed in a Lo-Bind Eppendorf tube. Any trapped peptides were extracted from the gel pieces by sonication in 40µL 50% (v/v) acetonitrile, 5% (v/v) formic acid. The resulting supernatant was added to the first before being concentrated to a final volume of 20µL using vacuum centrifugation [22].

2.5 LC-MS/MS analysis

2µL of each sample (4 biological replicates with 3 technical replicates each) were injected on a 1.0mm x 5 mm PepMap 100 C_{18} trap column, 5µm particle size, at a flow rate of 25µL/min then onto a 75 µm x 50 cm PepMap C_{18} column, 3µm particle size, at a flow rate of 300nL/min using a Dionex Ultimate^{TM} 3000 RSL nano system (Thermo Fisher Scientific, Massachusetts, USA). The mobile phase was 3% acetonitrile, 0.1 % formic acid in MS grade H_2O. Peptides were eluted using a linear gradient from 3-30% acetonitrile, 0.1% formic acid over 55 minutes. The peptides eluted from the column were analysed using a Q Exactive Plus mass spectrometer with a Nano Flex ionization source operating with Xcalibur acquisition software (Thermo

Fisher Scientific, Massachusetts, USA). The mass spectrometer was externally calibrated and operated in data-dependent mode. Full MS1 scans were acquired over a mass range of 375-1,500 m/z with a resolution setting of 70,000, while fragment ion spectra were acquired at a resolution of 17,500. For data dependent acquisition of HCD spectra, the top ten most intense ions were selected for fragmentation in each scan cycle and full MS and fragment ion spectra were detected by the Orbitrap mass analyser. Exclusion conditions were optimised according to the observed peak width (typically 10s).

2.6 Protein Identification

Processing of the raw data generated from LC-MS/MS analysis was carried out using Proteome Discoverer version 2.2 (Thermo Fisher Scientific; Massachusetts, USA). For the analysis, the grain, grain-corium junction and corium data from each extraction were combined. The following search parameters were used for protein identification: peptide mass tolerance 10 ppm, MS/MS mass tolerance 0.02 Da, up to two missed cleavages allowed, minimum peptide length, six amino acids, carbamidomethylation of cysteine was set as a fixed modification and oxidation of methionine, lysine and proline, acetylation of the N-terminal residue, and galactosyl, glucosylgalactosyl modifications of lysine were set as variable modifications. For each protein, the minimal number of unique peptides identified was set to two and the false discovery rate was set at 1%. The number of proteins initially identified was reduced from over 10,000 to approximately 1,000 using these criteria. Data were searched against the UniProtKB-SwissProt database (taxonomy: Bovine, release 10/2016).

2.7 Statistical analysis

Analysis of each sample was performed in three separate experiments. Statistical differences between the groups OSP and DA were determined using one-way student *t*-tests and volcano plots. In order to be classified as significantly different the p-value had to be less than 0.05 and the fold change greater than 2. Data analysis *via* principal component analysis (PCA) plots and visualization *via* heatmaps was carried out using the publicly available MetaboAnalyst 4.0 software (https://www.metaboanalyst.ca/).

3. Results and discussion

3.1 Proteomic profiles of hide during early leather processing stages

Proteins were extracted from hide, sampled during the four early leather processing stages: before processing - raw hide (A), after dehairing and liming (B), after deliming and bating (C) and after pickling (D), using a traditional lysis extraction buffer. Figure 5 illustrates how during the early leather processing stages the majority of low molecular weight bands, below 75kDa, are removed. During early leather processing stages the majority of non-collagenous proteins are removed whilst collagen is retained, thus these proteins are likely non-collagenous proteins [1]. The biggest change is between samples A and B indicating that the majority of these proteins are removed by the dehairing and liming process. Dehairing removes the hair from the hide using a combination of alkali and reducing agents which break down the sulphur-sulphur linkage in cysteine, a feature of keratin which is the major protein found in hair [1, 13]. Liming uses chemicals similar to those found in dehairing. It uses a combination of chemical and physical action to affect the protein in hide particularly collagen. It causes hydrolysis of amide groups, modification of guanine groups, hydrolysis of keto-imide links in protein chains, swelling and removal of unwanted material such as globular proteins and other interfibrillary substances [1, 13].

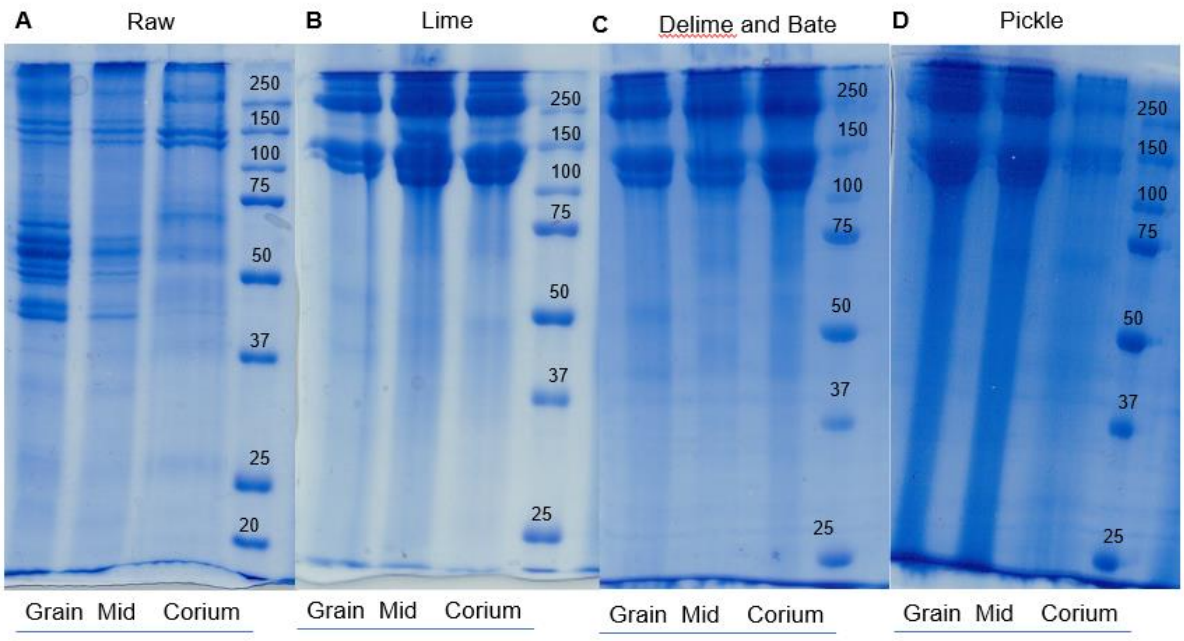

Figure 5. SDS-PAGE gels illustrating the proteomic profile of A) raw hide, B) limed hide, C) delime and bated hide and D) pickled hide extracted using lysis buffer.

Streakiness and overly dark lanes were predominately present in samples B, C and D, this is potentially due to the chemicals used during leather processing, this interfered with consistent results. The images also illustrate more intense bands in stages B, C and D even though the same concentration was loaded onto each well. This is potentially due to two reasons; firstly, re-solubilising the proteins after extraction proved difficult. Different techniques including the buffer utilised, heating and sonication were considered and/or tried however most proved ineffective. Which could lead to inconsistent and/or inaccurate results. Secondly, Bradford assays were used to determine the concentration of the samples. However, this method is unreliable with collagen, a major component of our samples [23, 24]. Other methods were considered to more accurately measure the concentration however most reacted unfavourably to the buffer that the proteins were solubilised in. Which could lead to incorrect protein concentrations being measured which would affect intensities on the gel.

3.2 Proteomic profile of loose and tight samples

Looseness can only be assessed in the finished leather; thus, two different areas of cattle hide were investigated, the official sampling position (OSP) located near the lower backbone and the distal axilla (DA) located in the rear armpit as shown in Figure 1.

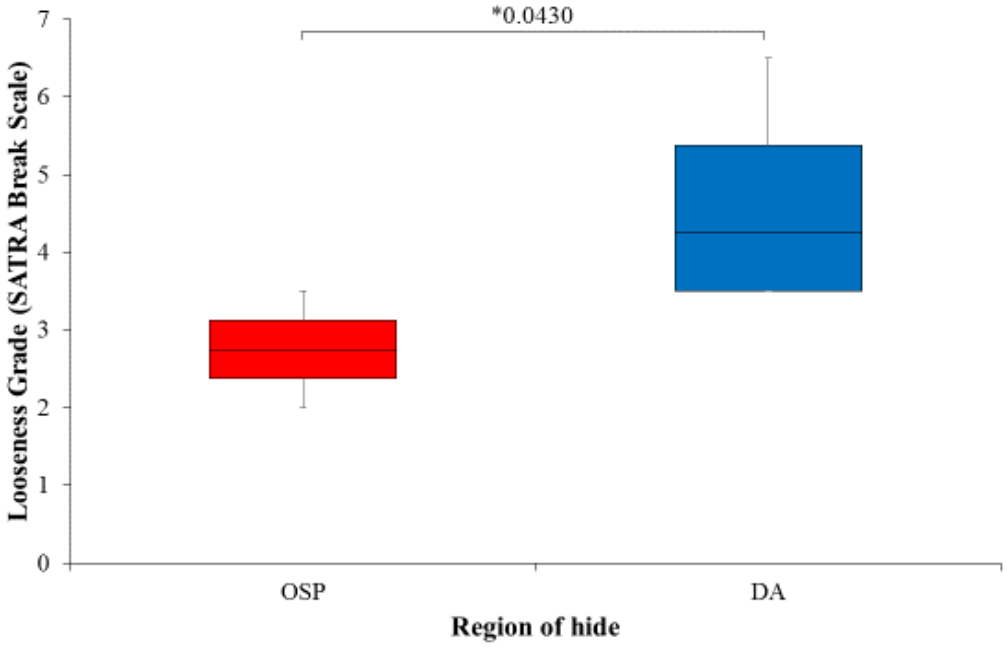

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Figure 6. Looseness grade of OSP and DA region of leather samples, *p-value.

These regions have shown variation in their mechanical properties [25], laxity and extensibility [25, 26] as well as topology, pH, temperature, moisture and microbiology [27] and prior studies have indicated that the DA region is more prone to the defect called looseness [2]. This study also shows significant difference between samples taken from the OSP and DA regions as shown in Figure 6.

The proteomic profiles of loose and tight cattle hide throughout the different stages were examined first using SDS-PAGE gels. Figure 7 illustrates the difference between loose and tight samples from raw hide whilst Figure 8 shows the difference between loose and tight samples from limed hide. The overall pattern of bands in tight and loose samples in both the raw and lime hide were very similar. Although in both stages slight differences were detected in the banding pattern especially below 75kDa, this is particularly clear in the limed stage which shows more of these proteins in the OSP region compared to the DA region. No differences were detected in the delime/bate (C) or pickle (D) samples (figure not shown). However, these differences as well as the differences in the intensities of certain bands tended to be inconsistent between gels and replicates and could be an issue related to the solubility or concentration of the samples as mentioned above.

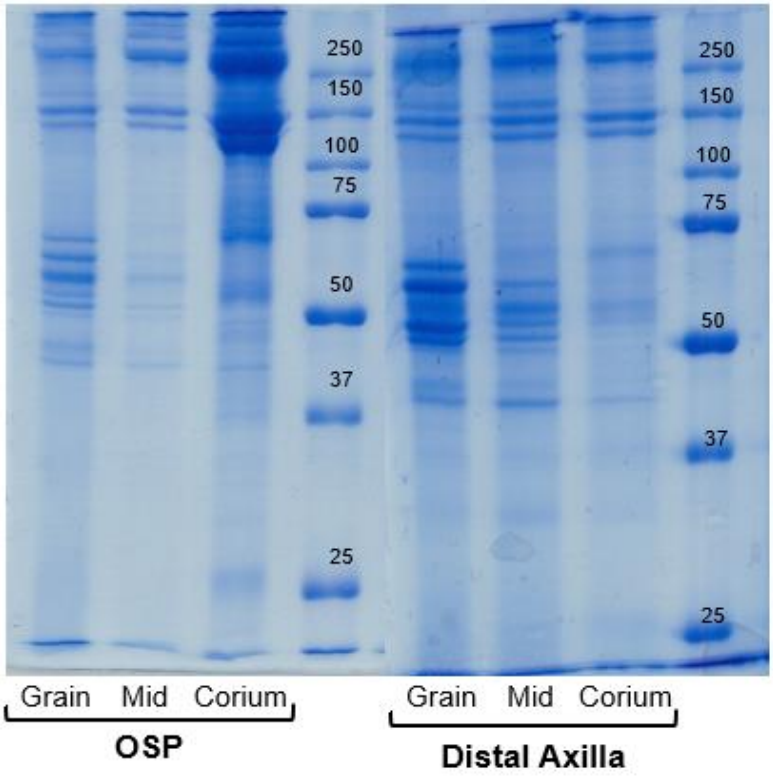

Figure 7. SDS-PAGE gel illustrating the proteomic profile of tight (OSP) and loose (DA) samples extracted from raw hide.

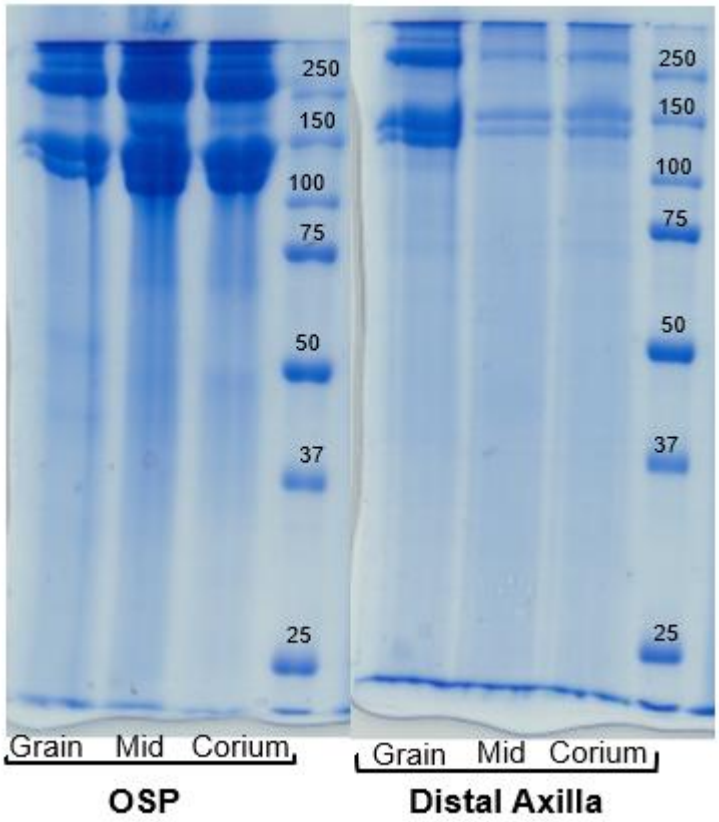

Figure 8. SDS-PAGE gel illustrating the proteomic profile of A) tight (OSP) and B) loose (DA) samples extracted from lime hide.

3.3 Proteomic analysis of raw hide using LC-MS/MS

In-gel LC-MS/MS was utilised to further analyse the proteins extracted from loose and tight samples. Due to the time constraints only an in-depth analysis of loose and tight raw hide samples was accomplished. The proteomic analysis identified 439 proteins with high confidence from the OSP region whilst samples from the DA region yielded 868 identifications (Figure 9a). Interestingly, the proteins that were common to both the OSP and DA regions (399 identified proteins) were typically up-regulated in the OSP region suggesting that regardless of the more complete extraction of proteins from the DA region there are higher concentrations of these of these proteins in the OSP region compared to the DA as shown in Figure 9b.

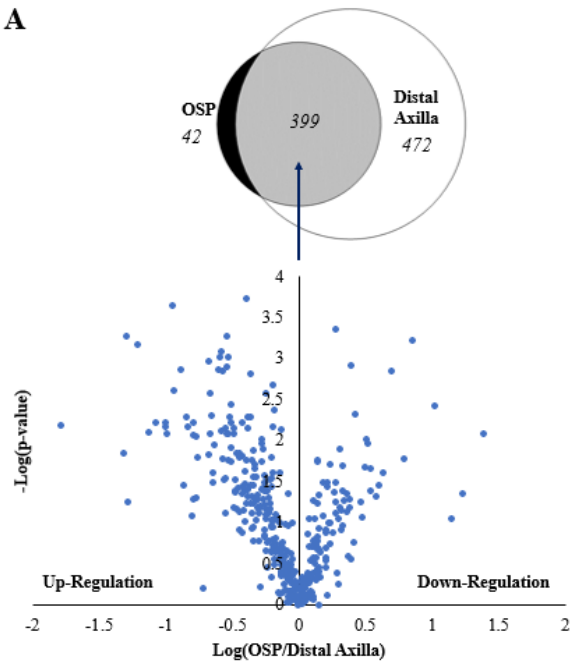

Figure 9. A) Venn diagrams comparing the number of proteins identified in OSP and DA samples and B) volcano plots comparing the statistical significance *vs* fold change in the abundance of proteins found in the OSP and DA for lysis extraction.

All proteins that were common to both the OSP and DA region were further analysed to determine whether there were any significant differences between the relative concentrations in the two regions. In order to be categorised as significantly different the proteins had to have p-values below 0.05 and a fold change equal to or greater than 2. Only 38 proteins met these constraints and all were down-regulated in the DA samples. These proteins included fibrous collagen, proteoglycans and other ECM proteins, keratins, cellular proteins, enzymes and serum proteins and are listed in Table 1.

Fibrous collagens type I and III are down-regulated in DA samples (Table 1). Fibrous collagen is the most abundant collagen in hide and provides mechanical and structural support to the hide with type I being more prevalent in the corium and type III in the grain [28, 29]. A decrease in the fibrous collagen may result in a less organised collagen network which could contribute to the development of looseness. Previous studies have indicated that loose leather has increased gaps between the collagen fibres [2-4] which could be attributed to a decreased concentration of fibrous collagen.

The proteoglycan decorin and proteoglycan associated protein glial hyaluronate binding protein are also down-regulated in DA samples (Table 1). The core protein of decorin interacts with specific surface amino acid residues on type I collagen fibrils, the interaction being stabilised by electrostatic interactions between collagen and the sulfates of the GAG [30]. This interaction is necessary for assembly of collagen microfibrils and prevents the cleavage of collagen fibrils by matrix metalloprotease I [30]. The glial hyaluronate binding protein is believed to be a proteolytic product of versican [31]. Versican is a hyalectan that binds to both hyaluronic acid and lectins and has roles in regulation of cell adhesion, migration and proliferation, extracellular matrix assembly and fibrillogenesis of elastic fibres [31]. As such, lower concentrations of these proteins could affect collagen fibre bundle architecture leading to looseness.

As seen in table 1 many keratins were down-regulated in the OSP compared to the DA. As keratins are removed during the dehairing stage of leather processing it is unlikely that they contribute to looseness. It is therefore possible that the difference in the concentration of keratins could also be due to sampling issues. However, it must be noted that in cells of the dermis, keratin filaments and other intermediate filaments function as part of the cytoskeleton to mechanically stabilise the cell against physical stress. So, a decrease in these filaments could cause a less mechanically stable cell structure. Other cellular proteins that have a role in supporting the structure of dermal cells include annexin, tubulin and myosin.

Of the enzymes that were down-regulated in the DA region, protein-lysine-6-oxidase was of the most interest. It is an enzyme essential for the formation of crosslinks between tropocollagen molecules as well as various extracellular matrix proteins including elastin [32]. Down regulation of this enzyme could result in defective fibrillogenesis leading to increased gaps and a less organised structure of collagen fibres. There was also a decrease in the lysosomal enzyme cathepsin C which activates serine proteases as well a decrease in the serine protease inhibitors alpha 1 anti-proteinase and isoform X2 of Serpin B6.

Table 1: Proteins that are significantly down regulated in the DA

	Protein	Accession	p-value	OSP/DA	Extraction Method
Fibrous Collagen	Collagen type I: alpha 1	AAI05185.1	0.0396	1.99	NU
	Collagen type 1: alpha1 CN8	0910139A	0.0474	3.77	L
	Precursor of collagen type III: alpha-1	NP_001070299.1	0.0213	3.15	L
Proteoglycans and ECM proteins	A Chain A, Decorin	1XCD	0.0204	2.11	L
	Glial hyaluronate-binding protein	AAB20399.1	0.0381	3.61	NU
Keratin	Keratin 31	DAA18488.1	0.0239	4.27	L
	Keratin 82	DAA29986.1	0.0216	3.46	L
	Keratin 84	DAA29999.1	0.0409	3.46	L
	Keratin 86	DAA30000.1	0.0144	1.98	NU
	Keratin 83	AAI23472.1	0.0037	10.54	L
	Keratin I: cytoskeletal 27	DAA18462.1	0.0006	7.10	L
	Keratin I: cytoskeletal 39	XP_010814574.2	0.0083	24.44	L
Cellular proteins	A Chain A, Actin, Cytoplasmic 1	3UB5	0.0047	2.67	L
	Actin, gamma-enteric smooth muscle	NP_001013610.1	0.0147	2.64	NU
	Annexin I	AAB25084.1	0.0441 0.0412	16.87 3.31	L NU
	Histone H2B type 1-K	DAA16155.1	0.0014	5.00	L
	Myosin-11	NP_001095597.1	0.0065	13.23	NU
	Isoform X1 of Periostin	XP_005213601.1	0.0045	7.29	NU
	Isoform X13 of Tropomyosin alpha-1 chain	XP_024853024.1	0.0164	4.89	NU
	Isoform X3 of Tropomyosin beta chain	XP_005210126.1	0.0138	3.77	NU
	Tubulin alpha 1C chain-like	XP_024838025.1	0.0049	23.66	NU
	Tubulin alpha 4a	AAI18200.1	0.0138	2.82	NU
	Tubulin beta 4B chain	NP_001029835.1	0.0011	13.34	NU
	Isoform X1 of V-set and immunoglobulin domain-containing protein 8	XP_010801062.1	0.0096	3.20	L
Enzymes	ADP/ATP translocase 3	DAA33747.1	0.0410	2.11	L
	Alpha-1-antiproteinase	P34955.1	0.0039	2.81	NU
	Bovine Mitochondrial F1-Atpase	2W6F	0.0054	14.72	NU
	Cathepsin C	AAI02116.1	0.0127	2.01	L
	Fatty acyl-CoA reductase 2	DAA29455.1	0.0353	3.99	L
	Precursor of Protein-lysine 6-oxidase	DAA27688.1	0.0164	6.13	L
	Pyruvate Kinase 2	AAI02827.1	0.0107	2.08	NU
	Isoform X2 of Serpin B6	XP_015315506.2	0.0004	10.37	NU
Serum Proteins	A Chain A, Bovine Fab E03 Light Chain	5IJV	0.0102	5.22	NU
	Albumin	754920A	0.0415	6.26	NU
	Alpha-2-macroglobulin	Q7SIH1.2	0.0039	18.34	NU
	Isoform X1 of Complement component C8 gamma chain	XP_005213573.2	0.0012	2.44	L
	Precursor of Complement component C9	NP_001030441.1	0.0431	2.30	L
	Immunoglobulin J chain	AAB03643.1	0.0105	3.31	L

4. Conclusion

In this study, the proteomic profiles of loose and tight cattle hide were investigated throughout early processing stages using gel LC-MS/MS. The DA region of hide was used as a model for loose hide, with the OSP region being used as a control. Proteins were extracted using a traditional lysis buffer and run on SDS-PAGE gels. Clear differences in the protein patterns were observed over the four different processing stages; raw hide, limed hide, delime/bated hide and pickled hide. With raw hide containing many proteins which were not present in the lime, delime/bate or pickle samples. Likely due to the removal of non-collagenous proteins in these stages. However only subtle and inconsistent differences were detected between loose and tight samples using this method. LC-MS/MS was used to further identify these proteins and examine any differences in protein or concentration. Due to time constraints raw hide was the only stage that was further studied. Over 400 proteins were identified in raw hide with high confidence, and there were clear differences between the two regions tested some of which provided a molecular explanation for the differences in appearance of loose leather from tight leather. It was particularly interesting that four of the proteins that were significantly down regulated in the DA are involved in or influence the arrangement of collagen microfibre bundles that are responsible for the physical properties of the hide. Although these results need to be validated, the preliminary studies indicate that there are molecular differences in the raw hides that produce loose and tight leather. Understanding the molecular causes of loose leather may enable biomarkers to be developed for its early detection.

5. Future Directions

This preliminary work to investigate the molecular cause of looseness using gel-LC-MS/MS has provided a greater understanding of how proteins affect the quality of leather, particularly in response to defects. Studying the proteomic profiles in the different stages including lime, delime/bate and pickle would enable a comprehensive understanding of how the differences in raw material affect the final leather product. Further validation of the findings through techniques such as western blotting or ELISA could lead to biomarkers being developed for the early detection of looseness. This technique could also be utilised for other leather defects of interest.

6. Acknowledgements

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Investigating the proteomic profiles of cattle hide resulting in loose and tight leather throughout early processing stages

Globally, looseness is a defect found in cowhides and causes a wrinkly appearance in the finished leather reducing its quality. The molecular-microstructure relationships between loose and tight cowhides are yet to be explored which will provide valuable and accessible information for the industry to refine hide processing protocols to produce leather with improved physical properties.

Catherine Maidment has investigated the underlying cause of cowhide looseness using proteomics to analyse the protein composition of loose and tight cowhides. This work presented and for the first on the nano-LC-MS/MS protein profile in loss and tight cowhides. The proteins were extracted from the cowhide using lysis buffer then tryptic digested before being injected on the nano-LC-MS/MS. The processing of the raw data was performed using Proteome Discoverer version 2.2. Several hundred proteins were identified in cowhides, many of which were more present in the distal axilla than the official sampling position.

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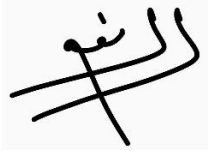

Kind Regards
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